AROMATIC HYDROCARBONS: DEGRADATION BY BACTERIA AND FUNGI.

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The decline in the supply of natural oil and gas reserves over the past 10 years has caused the Western Hemisphere to import huge volumes of petroleum and petroleum products. If the Western Hemisphere persists in maintaining a petroleum based industrial economy alternative sources of energy will have to be found and developed.

Oil Shale

One alternative source of energy is the extraction of the organic components of oil shale to yield oil and gas. Numerous organic rich shale deposits occur throughout the United States. The release of the energy yielding organic fraction from oil shale has been brought about by retorting the oil shale at temperatures above 500° C and distillating the organic material. This pyrolytic process also generates solid waste in the form of spent oil shale ash. A part of this oil shale ash consists of polycyclic aromatic hydrocarbon derivatives such as benzo[a]-anthracene, dibenzo[a,h]anthracene, benzo[a]pyrene and 3-methylcholanthrene. These aromatic hydrocarbons can be metabolized by mammals and have been implicated in chemical carcinogenesis.

It is known that aromatic hydrocarbons are formed by pyrolytic processes. The types of compounds that are produced depends on the temperature of formation. Thus, the low temperatures that are associated with petroleum formation produce a diverse array of aromatic structures. A

predominant feature of this group of compounds is the presence of alkyl substituents. In contrast, pyrolysis at high temperatures leads to the formation of unsubstituted aromatic hydrocarbons.

Fate in the Environment

Crude petroleum is introduced into the environment by natural means (oil seeps) or by the activities of man. Factors that affect the oil include evaporation of volatile components, dissolution, photochemical and biological degradation. The latter process has been amply demonstrated with respect to the linear and branched alkane components of petroleum.

For many years it has been tacitly assumed that crude petroleum in the reservoirs is immune to microbial attack. However, recent studies have shown that under appropriate conditions extensive degradation can occur. It is possible that microbial activities play a significant role in the formation of tar sands.

Little is known about the microbial degradation of the aromatic constituents of petroleum. This is not surprising in light of the immense numbers of different structures that are known to be present. Nevertheless, studies with relatively simple aromatic hydrocarbons have revealed general features of biodegradation that may be used to predict the fate of more complex molecules.

Microbial Degradation

Oil pollutants are recycled in nature by the metabolic activities of microorganisms. The environment contains a wide range of different bacterial species, filamentous fungi and yeasts that degrade hydrocarbons. The rate of degradation of crude oil is markedly influenced by a range

of environmental conditions. Some of the more significant factors are organism availability, nutrient availability, oxygen, temperature, and type of petroleum.

There is a specificity of the utilization of individual hydrocarbons by different microorganisms depending upon the enzyme repertoire of the organisms involved and the molecular conjugation of the hydrocarbon. It has been demonstrated that hydrocarbon-utilizing bacteria have a preference for long chain aliphatic hydrocarbons. Straight chain n-alkanes containing 10-18 carbon atoms are assimilated with the greatest frequency and rapidity. Branched chain alkanes, monocyclic substrates and aromatic compounds are utilized least frequently.

Crude oil contains thousands of aromatic hydrocarbons and related compounds. Relatively few of these molecules have been studied in terms of metabolism by microorganisms and higher forms of life. Nevertheless it now appears that certain general features of degradation have emerged which distinguish the mechanisms used by procaryotes and eucaryotes to degrade the benzenoid nucleus.

Mammalian Metabolism

It has been well documented that mammals metabolize aromatic hydrocarbons via the oxidative formation of arene oxides. This reaction is catalyzed by a monooxygenase and is characterized by the incorporation of one atom of molecular oxygen into the hydrocarbon substrate. The enzyme system involved in this hydroxylation is membrane bound and functions as a multicomponent electron transport system for a variety of endogenous substrates. It has an absolute requirement for NADPH and molecular oxygen and is also known as microsomal hydroxylase, or a mixed function oxidase.

Mammals metabolize naphthalene to form naphthalene 1,2-oxide. The 1,2-naphthalene oxide formed can spontaneously isomerize to 1-naphthol by a reaction that has been termed the NIH shift. Alternatively, the 1,2-naphthalene oxide can undergo enzymatic hydration by the enzyme epoxide hydrase to yield *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (*trans* naphthalene dihydrodiol). Another reaction of the naphthalene oxide is conjugation both spontaneously and by enzymatic catalysis with glutathione which leads to the formation of mercapturic acids.

Bacterial Metabolism

Until recently bacteria were thought to metabolize aromatic hydrocarbons through *trans*-dihydrodiols. Investigations in our laboratory implicated *cis*-3,5-cyclohexadiene-1,2-diol (*cis*-benzene diol) as an intermediate in the oxidation of benzene by *Pseudomonas putida*. Bacteria incorporate two atoms of molecular oxygen into aromatic hydrocarbons to form *cis*-dihydrodiols. *cis*-Dihydrodiols have now been implicated as intermediates in the bacterial metabolism of a variety of aromatic hydrocarbons and related compounds. Further oxidation yields catechols which are the substrates for enzymatic fission of the aromatic ring. *Pseudomonas putida* and *Pseudomonas* species NCIB 9818 form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) when grown in the presence of naphthalene. This reaction is a prerequisite for subsequent fission of the aromatic nucleus.

Fungal Metabolism

There have been several studies on the metabolism of aromatic hydrocarbons by fungi. These investigations suggest a biochemical relatedness between fungal and mammalian liver microsomes towards the monooxygenation of aromatic substrates.

The fungi used in this study were isolated by enrichment culture technique. Soil and water samples were obtained form littoral areas along the coast of North Carolina and placed in a crystallization dish. Crude oil was added to the water surface and the dishes were incubated for 3-4 weeks at 25° C. After this incubation period, samples were removed and streaked on well plates. The well plate procedure was developed as a method for adding hydrophobic substrates to agar plates in a way that growth could readily be observed. Fungal growth predominated and several different genera of fungi were isolated.

The fungus used throughout this report was identified and characterized as *Cunninghamella elegans*, a terrestial phycomycete.

This organism can utilize a wide variety of paraffinic and mixed base crude oil as sole source of carbon and energy. In addition, *C. elegans* can utilize naphthalene, all alkanes tested from C_3 through C_{32}, a wide variety of long chain alkenes, ketones and fatty acids as sole source of carbon and energy.

Cells of *Cunninghamella elegans* were incubated with naphthalene for 24 hours. After 24 hours the mycelia was filtered and the culture filtrate extracted with three volumes of ethyl acetate. Thin layer chromatographic analysis of the culture filtrate revealed the presence of six transformation products. Each product absorbed ultraviolet light and gave a characteristic color with Gibb's reagent. In order to ascertain that the products were indeed derived from naphthalene the experiment was repeated with ^{14}C-naphthalene. Six radioactive products were observed that corresponded to the metabolites located on thin layer chromatograms by Gibb's reagent.

To isolate the transformation products *C. elegans* was grown in the presence of 4 liters of dextrose broth in the presence of 2 grams of naphthalene. After 24 hours the culture filtrate was extracted with ethyl acetate. Removal of the solvent gave 1.54 grams of a brown solid that was applied to a deactivated silica gel column (20% water). The column was eluted with chloroform and 20 ml fractions were collected. After 30 fractions had been collected the eluting solvent was changed to chloroform:methanol (99:1). Products eluting from the column were detected by thin layer chromatography in solvent A (chloroform:acetone 80:20).

The residue obtained from fractions 5-8 was recrystallized to leave 37 mg of yellow needles. Preparative thin layer chromatograhy of the mixture gave 15 mg of pure 1,4-naphthoquinone and approximately 1 mg of 1,2-naphthoquinone. The isolated 1,4-naphthoquinone gave identical melting point, infrared, proton magnetic resonance, mass and ultraviolet spectra to those given by a freshly sublimed sample of synthetic 1,4-naphthoquinone. The isolated 1,2-naphthoquinone gave an identical absorption spectrum and melting point to that given by authentic 1,2-naphthoquinone. The residue from fractions 9-17 was sublimed under vacuum at 50° C for 4 hours. The sublimed product (776 mg) was obtained in the form of needle-shaped white crystals that melted at 96-98° C. This melting point was not depressed on admixture with a pure sample of synthetic 1-naphthol. Also 2-naphthol was obtained in pure form by preparative thin layer chromatography.

In order to ascertain the ratio of 1- to 2-naphthol produced by *C. elegans* a crude sample of the transformation products was treated with trifluoroacetic anhydride and analyzed by gas chromatograhy. The ratio was found to be 96:4 (1-naphthol:2-naphthol).

Fractions 18-27 were pooled and the solvent removed to leave 297 mg of a yellow oil. The oil was dissolved in a small volume of chloroform and applied to a column of neutral alumina. The column was eluted with chloroform and 5 ml fractions were collected. Fractions 3-10 were pooled and removal of solvent gave 265 mg of a yellow oil. This material was chromatographed and showed the presence of a single compound (R_f, 0.38). The infrared, proton magnetic resonance, mass and ultraviolet spectra established the structure of the compound as 4-hydroxy-1-tetralone. Additional evidence was provided by treating this compound with chromic acid. The resulting oxidation product gave an identical ultraviolet absorption spectrum and was chromatographically identical to synthetic 1,4-naphthoquinone.

The residue obtained from fractions 45-64 was recyrstallized from petroleum ether. Two recrystallizations gave 63 mg of white needles that melted at 104-105° C. This melting point was not depressed on admixture with a pure sample of synthetic *trans*-1,2-dihydroxy-1,2-dihydronaphthalene. In addition acid catalyzed dehydration (6N HCl, 100° C, 15 min) of the dihydrodiol produced material with the ultraviolet spectrum and chromatographic properties of 1-naphthol.

High pressure liquid chromatography was employed to separate and quantitate each metabolite produced from naphthalene by *C. elegans*. The major metabolites were 1-naphthol (67.9%) and 4-hydroxy-1-tetralone (16.7%). Minor products isolated were 1,4-naphthoquinone (2.8%), 1,2-naphthoquinone (0.2%), 2-naphthol (6.3%) and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene (5.3%).

Centrifugation studies on mycelia extracts of *C. elegans* indicated that naphthalene hydroxylase activity was located in the 10,000 x g supernatant fraction. High speed centrifugation (100,000 x g for 1 hr)

of the 10,000 x g supernatant yielded a pellet with 95% of the naphthalene hydroxylase activity. The metabolites formed from the microsomal hydroxylation of naphthalene were *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol and 2-naphthol. The amount of naphthalene hydroxylase activity remaining in the 100,000 x g supernatant varied from 1-5% but was generally very low. The microsomal hydroxylation of naphthalene by *C. elegans* was found to specifically require NADPH. When NADH was substituted as a source of reducing power no activity was observed. The enzyme system was inhibited by carbon monoxide, SKF-525A, and metapyrone implicating that naphthalene hydroxylation in *C. elegans* may involve a factor similar to cytochrome P-450. These results were further supported by the lack of inhibition when treated with cyanide ions.

The data presented in this paper indicates that the fungus *C. elegans* metabolizes naphthalene similar to monooxygenases of hepatic microsomes. There is relatively little information on the microbial degradation of aromatic hydrocarbons when the molecules are present as constituents of crude oil. Nevertheless it is possible that the types of reactions described do occur when microorganisms interact with oil in the environment.